Toll-like Receptor Expression Profile of Human Dental Pulp Stem/Progenitor Cells

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Abstract

Introduction: Human dental pulp stem/progenitor cells (DPSCs) show remarkable regenerative potential *in vivo*. During regeneration, DPSCs may interact with their inflammatory environment via toll-like receptors (TLRs). The present study aimed to depict for the first time the TLR expression profile of DPSCs. **Methods:** Cells were isolated from human dental pulp, STRO-1–immunomagnetically sorted, and seeded out to obtain single colony-forming units. DPSCs were characterized for CD14, CD34, CD45, CD73, CD90, CD105, and CD146 expression and for their multilineage differentiation potential. After incubation of DPSCs in basic or inflammatory medium (interleukin-1β, interferon-γ, interferon-α, tumor necrosis factor-α), TLR expression profiles were generated (DPSCs and DPSCs-i). **Results:** DPSCs showed all characteristics of stem/progenitor cells. In basic medium DPSCs expressed TLRs 1–10 in different quantities. The inflammatory medium upregulated the expression of TLRs 2, 3, 4, 5, and 8, downregulated TLRs 1, 7, 9, and 10, and abolished TLR6. **Conclusions:** The current study describes for the first time the distinctive TLR expression profile of DPSCs in uninflamed and inflamed conditions. *(J Endod 2016;42:413–417)*

Key Words

Flow cytometry, polymerase chain reaction, pulp, stem cells, TLR

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The pulp-dentin complex stems embryonically from the multipotent neural crest–ectomesenchyme and constitutes a functional and physiological unit (1). As a highly vascularized and innervated connective tissue, it is composed of diverse cell populations, among which dental pulp stem/progenitor cells (DPSCs) are anticipated to continuously replenish odontoblasts to form secondary and tertiary/reparative dentin throughout adult life as well as in response to various insults (2). DPSCs are considered an alternative and readily available source of multipotent stromal cells (MSCs) for tissue regeneration; they are characterized by their clonogenicity, their highly proliferative potential, their capability of self-renewal, and their multilineage differentiation aptitude (3) and are reported to hold promising attributes in the field of ameliorating ischemic, cardiac, and neurologic diseases (4, 5).

Toll-like receptors (TLRs), key molecules connecting innate and adaptive immunity, are germline encoded pattern recognition receptors sensing specific pathogen-associated molecular patterns (PAMPs), thereby promoting activation of immune cells, and are pivotal in pathogenesis of chronic inflammatory, autoimmune, and infectious diseases (6). To date, 10 functional human TLRs have been described. Depending on their cellular localization and PAMP ligands, TLRs are divided into extracellular ones, mostly identifying microbial membrane constituents including lipids and lipoproteins (TLR1, TLR2, and TLR6), lipopolysaccharide (LPS) (TLR4), and flagellin (TLR5), and intracellular ones, which recognize double-stranded RNA (TLR3), single-stranded viral RNA (TLR7 and TLR8), and unmethylated CpG-DNA of viruses and bacteria (TLR9) (7).

MSCs of different origin express functional TLRs in characteristic patterns, making them particularly sensitive to certain microbial compounds. When activated by their ligands, TLRs modulate the migratory, proliferative, differentiation, and immunosuppressive potentials of MSCs (8). Varied expressions of TLRs 1, 2, 3, 4, 5, and 6 were reported on human and mural adipose MSCs and bone marrow–derived MSCs (BM-MSCs) as well as on human Wharton jelly MSCs (WJ-MSCs) (9). This distinctive TLR expression pattern could affect the therapeutic potential of MSCs during transplantation *in vivo* (10). To date, solely the expression of TLRs 2, 3, and 4 was described on DPSCs (11–14). The aim of the present investigation was to characterize for the first time a complete TLR expression profile of DPSCs under inflamed and uninflamed conditions.

Materials and Methods

Isolation and Culture of DPSCs

Human dental pulp tissue was obtained from patients (age, 15–20 years) undergoing extraction of non-carious third molars (n = 6) (Institutional Review Board approval number D-444/10). Teeth were disinfected and mechanically fractured, and the dental pulp was gently isolated with sterile forceps, rinsed several times in basic medium, and placed into 75-mL culture flasks (Sarstedt AG, Nümbrecht, Germany) for 30 minutes to adhere. Subsequently, the basic medium was carefully added, flasks were incubated in 5% carbon dioxide at 37°C, and cells were left to grow out.

After reaching 80%–85% confluence, cells were detached with 0.10% trypsin-EDTA (Biochrom Ltd, Cambridge, UK) and counted; their viability was tested by using trypan blue (Sigma-Aldrich, St Louis, MO) and finally seeded out at 30 cells/cm^2 density in basic medium. After the first passage cells reached 80%–85% confluence, they were immunomagnetically sorted by using anti-STRO-1 (BioLegend, San Diego, CA) and anti-

immunoglobulin M MicroBeads (Miltenyi-Biotec, Bergisch Gladbach, Germany) antibodies according to the manufacturer's instructions (MACS; Miltenyi-Biotec). The STRO-1$^+$–sorted cells (DPSCs) were seeded out to form colony-forming units (CFUs).

CFUs

DPSCs were cultured in basic medium at 1.63 cells/cm^2 density. Aggregates of 50 or more cells were scored as CFUs. On day 12, representative cultures were fixed with 4% formalin and stained with 0.1% crystal violet. The remainder of the CFUs were detached by using cell scrapers and seeded in new 75-mL flasks.

Flow Cytometric Analysis

After reaching confluence, DPSCs were characterized flow cytometrically for the MSCs' surface markers: CD14, CD34, CD45, CD73, CD90, CD105, and CD146 (all from Becton Dickinson Co, Canaan, CT). Binding of primary antibodies and isotype controls was performed by using FcR Blocking Reagent (Miltenyi-Biotec), and their expression was evaluated with FACSCalibur E6370 and FACSComp 5.1.1 software (Becton Dickinson Co).

Multilineage Differentiation Potential

For osteogenic differentiation, third passage 2×10^4 DPSCs were cultured on 6-well plates in osteogenic medium (PromoCell, Heidelberg, Germany) and in basic medium (control). At day 14, cell cultures were stained with alizarin red (Sigma-Aldrich) to label calcified deposits. For adipogenic differentiation, third passage 3×10^5 DPSCs were cultured on 6-well plates in adipogenic medium (PromoCell) and in basic medium (control). The presence of lipid droplets was evaluated after 21 days by oil red O (Sigma-Aldrich). For chondrogenic differentiation, micromasses of third passage 3×10^4 DPSCs were incubated with chondrogenic medium (PromoCell) in 1.5-mL Eppendorf tubes (Eppendorf, Hamburg, Germany) and in basic medium (control). Chondrogenic differentiation was evaluated at day 35 by staining of glycosaminoglycans with alcian blue and nuclear fast red counterstaining (Sigma-Aldrich).

Inflammatory Medium

To test the effect of the inflammatory environment on the TLR expression profile of DPSCs, standardized inflammatory medium, composed of 25 ng/mL interleukin (IL)-1β, 1×10^3 U/mL interferon (IFN)-γ, 50 ng/mL tumor necrosis factor (TNF)-α, and 3×10^3U/mL IFN-α (inflammatory medium; all from PeproTech, Hamburg, Germany) (15) added to the basic medium components, was used. DPSCs were incubated 18 hours in the inflammatory (DPSCs-i) or basic medium (DPSCs).

mRNA Extraction and cDNA Synthesis

mRNA extraction was performed for DPSCs and DPSCs-i by using RNeasy kit (Qiagen, Hilden, Germany). Obtained RNA was purified by using RNase-free-DNase (Promega, Mannheim, Germany) and quantified photometrically. Complementary cDNA was synthesized from 1 to 13 μL RNA (1 μg/μL) by reverse transcription by using QuantiTect reverse transcription kit (Qiagen) in a volume of 20 μL reaction mixture containing 4 pmol of each primer, 10 μL of the LightCycler Probes Master mixture (Roche Diagnostics, Risch-Rotkreuz, Switzerland), and 5 μL specimen cDNA. Real-time polymerase chain reaction (rt-PCR) (LightCycler-96 Real-Time-PCR System; Roche Molecular Biochemicals, Indianapolis, IN) was performed according to the manufacturer's instructions. Relative quantities of each transcript were normalized according to the expression of phosphoglycerate kinase 1 (PGK1). Primers for TLRs 1–10 and PGK1 reference gene were supplied by Roche and tested on DPSCs and DPSCs-i (Table 1).

Flow Cytometric Determination of TLR Expression

DPSCs and DPSCs-i were characterized flow cytometrically for the expression of TLRs 1–10 at protein level. For intracellular TLR staining, cells were fixed and permeabilized with Fix&Perm kit (Imtec, Antwerpen, Belgium) before incubation. Antibodies used were anti-TLR1, anti-TLR3, and anti-TLR9 (eBioscience, San Diego), anti-TLR2, anti-TLR4, and anti-TLR8 (Enzo Life Sciences, Lörrach, Germany), anti-TLR5 (R&D Systems, Hessen, Germany), anti-TLR6 (BioLegend), anti-TLR7 (Perbio Science, Bonn, Germany), and anti-TLR10 (Acris Antibodies, Herford, Germany). Binding of primary antibodies and corresponding isotype controls was performed by using FcR Blocking Reagent and evaluated with FACSCalibur E6370 and FACSComp 5.1.1 software (Becton Dickinson).

Statistical Analysis

Shapiro-Wilk test tested the normal distribution of data. Differences in TLR expression on mRNA and protein levels in DPSCs and DPSCs-i were evaluated by using the Wilcoxon signed rank test (SPSS software version 11.5; SPSS, Chicago, IL). The level of significance was $P = .05$.

Results

Microscopy, CFUs, and Flow Cytometric Analysis

After the initial adherence phase, fibroblast-like cells grew out of the pulpal tissue masses (Fig. 1*A*). Twelve days after seeding, STRO-1$^+$–sorted cells (DPSCs) showed CFUs (Fig. 1*B*) and were CD14$^-$, CD34$^-$, and CD45$^-$ and CD73$^+$, CD90$^+$, CD105$^+$, and CD146$^+$ (Fig. 1*C*).

Multilineage Differentiation

The osteogenic differentiation of DPSCs was demonstrated by the formation of alizarin red–positive calcified deposits in contrast to their control (Fig. 1*D* and *E*). Adipogenic differentiation of DPSCs resulted in the formation of oil red O–positive lipid droplets in contrast to their control (Fig. 1*F* and *G*). The chondrogenic differentiation of DPSCs resulted in the formation of alcian blue–positive glycosaminoglycans in contrast to their control (Fig. 1*H* and *I*).

TLR mRNA Expression

On the mRNA level, DPSCs incubated in basic medium expressed (median gene copies/PGK1 copies, Q25/Q75) TLR1 (0.0013, 0.0008/0.0058), TLR2 (0.0002, 0.0000/0.0018), TLR3 (0.0015, 0.0009/0.0022), TLR4 (0.0067, 0.0024/0.0105), TLR5 (0.0000, 0.0000/0.0005), TLR6 (0.0017, 0.0007/0.0026), and TLR10 (0.0005,

TABLE 1. Primer Names and ID Used for rt-PCR (as supplied by Roche)

Assay ID	Gene symbol	Roche accession ID
111000	TLR1 H. sapiens	ENST00000308979
145617	TLR2 H. sapiens	ENST00000260010
111008	TLR3 H. sapiens	ENST00000296795
135752	TLR4 H. sapiens	ENST00000355622
103674	TLR5 H. sapiens	ENST00000366881
111018	TLR6 H. sapiens	ENST00000381950
111012	TLR7 H. sapiens	ENST00000380659
103816	TLR8 H. sapiens	ENST00000218032
143252	TLR9 H. sapiens	ENST00000360658
141065	TLR10 H. sapiens	NM_001017388
102083	PGK1 H. sapiens	ENST00000373316

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